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NACP, the precursor protein of the non-amyloid β/A4 protein (Aβ) component of Alzheimer disease amyloid, binds Aβ and stimulates Aβ aggregation

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ABSTRACT **NACP, a 140-amino acid presynaptic protein, is the precursor of NAC [the non-amyloid β/A4 protein (Aβ) component of Alzheimer disease (AD) amyloid], a peptide isolated from and immunologically localized to brain amyloid of patients afflicted with AD. NACP produced in *Escherichia coli* bound to Aβ peptides, the major component of AD amyloid. NACP bound to Aβ1–38 and Aβ25–35 immobilized on nitrocellulose but did not bind to Aβ1–28 on the filter under the same conditions. NACP binding to Aβ1–38 was abolished by addition of Aβ25–35 but not by Aβ1–28, suggesting that the hydrophobic region of the Aβ peptide is critical to this binding. NACP-112, a shorter splice variant of NACP containing the NAC sequence, bound to Aβ, but NACPδ, a deletion mutant of NACP lacking the NAC domain, did not bind Aβ1–38. Furthermore, binding between NACP-112 and Aβ1–38 was decreased by addition of peptide Y, a peptide that covers the last 15 residues of NAC. In an aqueous solution, Aβ1–38 aggregation was observed when NACP was also present in an incubation mixture at a ratio of 1:125 (NACP/Aβ), whereas Aβ1–38 alone or NACP alone did not aggregate under the same conditions, suggesting that the formation of a complex between Aβ and NACP may promote aggregation of Aβ. Thus, NACP can bind Aβ peptides through the specific sequence and can promote Aβ aggregation, raising the possibility that NACP may play a role in the development of AD amyloid.**

Alzheimer disease (AD) is a neurodegenerative disease neuropathologically characterized by the presence of senile plaques and neurofibrillary tangles (1). Protein aggregates that can be stained with thioflavine S and Congo red form the amyloid at the senile plaque core and in blood vessel walls (2). The major component of amyloid is β/A4 protein (Aβ), which consists of 39–43 amino acid residues derived from the much longer amyloid precursor protein (APP) (3). Aβ peptide is synthesized under physiological conditions in a soluble form that can be found in cerebrospinal fluid (4–6).

It is reported that synthetic Aβ peptides at high concentrations can form aggregates by themselves (7), and it is also suggested that these aggregates, rather than the monomeric form of the peptide, show toxicity to neuronal cells (8, 9). Several molecules detected in senile plaques, including proteoglycan (10), α_1-antichymotrypsin (11), the complement protein C1q (12), apolipoprotein E (13, 14), and apolipoprotein J (15), were shown to bind to the synthetic Aβ peptides *in vitro*. Also, transthyretin, a major cerebrospinal fluid protein, was found to bind Aβ (16). These proteins may interact with the Aβ peptide and may regulate amyloid formation and modulate Aβ neurotoxicity *in vivo* (17–19).

We identified an amyloid component in the AD brain (20) and named it non-Aβ component of AD amyloid (NAC). Immunohistochemical analysis of AD brains with polyclonal antibodies against NAC indicated that NAC localized to the amyloid of plaques and vessels. Molecular cloning of cDNA containing a sequence encoding NAC, from a human adult brain library, demonstrated that it is derived from a unique hydrophobic domain of the 140-amino acid precursor protein NACP (20). We have shown that NACP is relatively abundant in the frontal cortex, hippocampus, olfactory bulb, and striatum in the adult rat brain (21).

To study the potential involvement of NACP in amyloidogenesis in AD, we produced NACP in *Escherichia coli* and analyzed its binding to the Aβ peptides *in vitro*. NACP binds Aβ peptides through specific sequences and promotes Aβ aggregation.

MATERIALS AND METHODS

Materials. Aβ1–28 was purchased from Bachem Bioscience. Aβ1–38, Aβ1–35, and Aβ25–35 were synthesized by the fluoren-9-ylmethoxycarbonyl method. Peptide V (amino acids 1–9 of NACP), peptide X1 (amino acids 61–69 of NACP, which corresponds to the first 9 residues of NAC), and peptide Y (amino acids 81–95 of NACP, which corresponds to the last 15 residues of NAC) were synthesized as reported (20, 21) (see Fig. 1 for the location of each peptide). Affinity-purified rabbit anti-NACP polyclonal antibody, which recognizes the C-terminal region of NACP, was prepared as described (21). Anti-Aβ monoclonal antibody (10D5) was a generous gift of Athena Neurosciences (South San Francisco, CA).

Construction of Recombinant DNA. pGEX-2T vector was chosen to produce NACP as a fusion protein with glutathione *S*-transferase (GST). The cDNA encoding NACP (20) was digested with *Nco* I. A 1.65-kb NACP cDNA fragment was purified and treated with Klenow fragment to make both ends blunt, ligated with pGEX-2T that had been linearized with the restriction enzyme *Sma* I, and treated with calf intestinal alkaline phosphatase. *E. coli* K-12 DH5α cells were transformed with this ligation mixture, and ampicillin-resistant colonies were selected. A shorter splice variant of NACP, NACP-112 (22), was produced as a fusion protein with GST using pGEX-3X as a vector. The *Eco*RI fragment (1.09 kb) spanning the coding region of NACP-112 was inserted into pGEX-3X previously linearized with the same enzyme. *E. coli* DH5α cells were transformed with the resultant recombinant DNA, pGENACP-112. The NACPδ construct was produced as follows. Two oligonucleotide primers containing the *Bcl* I site were chosen based on the sequences 5′ and 3′ to NAC regions

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Abbreviations: Aβ, amyloid β/A4 protein; AD, Alzheimer disease; APP, Aβ precursor protein; NAC, non-Aβ component of AD amyloid; NACP, NAC precursor.

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of the NACP-112 cDNA: 5′-ttggtctgatcagccactg-3′ (δ1) and 5′-tcaaaaatgatcagttgggc-3′ (δ2), respectively. Two other oligonucleotide primers were designed based on the multiple cloning site sequence of pBluescript SKII plasmid: 5′-aaaagctggagctccaccg-3′ (BC1) and 5′-aattgggtaccgggcccc-3′ (BC2). Two separate PCRs were performed using δ1/BC1 primers, δ2/BC2 primers, and pBluescript SKII-NACP-112 as a template. δ1/BC1 primers produced the 5′ half of NACP-112 cDNA and δ2/BC2 primers produced the 3′ half of NACP-112 cDNA, with a gap in between. The products were purified on agarose gel, subjected to *Bcl* I restriction digestion, and ligated together at the *Bcl* I sticky ends. The ligated product was digested at the 5′ and 3′ ends with *Eco*RI, ligated into the PGEX-3X expression vector, and transformed into *E. coli* DH5α.

Production of NACP, NACP-112, and NACPδ. Cells were collected by centrifugation and resuspended in 40 ml of STE buffer (8% sucrose/50 mM Tris·HCl, pH 8.0/25 mM EDTA). The cell suspension was mixed with 4 ml of lysozyme (10 mg/ml) and incubated at room temperature for 15 min. Triton X-100 was then added to a final concentration of 1%, incubated at room temperature for an additional 15 min, and sonicated for 1 min three times to complete disruption of the cells. The lysate was centrifuged at 20,000 × *g* for 10 min at 4°C, and the supernatant was mixed with glutathione-agarose, preswollen in 50 mM Tris·HCl (pH 8.0), and rotated overnight at 4°C. The fusion protein was eluted with the same buffer containing the reduced form of glutathione (5 mM). After addition of $CaCl_2$ to a final concentration of 2.5 mM, 2 μg of human thrombin was mixed with 1 mg of the fusion protein and incubated at 25°C for 30 min. The reaction mixture was diluted to adjust the Tris·HCl and NaCl concentrations to 20 and 30 mM, respectively, and was applied to an MA7Q anion-exchange column (Bio-Rad) previously equilibrated with 20 mM Tris·HCl (pH 8.0). NACP was eluted with a linear gradient of NaCl (0–0.4 M). NACP-112 and NACPδ fusion proteins were cleaved with the other protease, factor Xa.

***In Vitro* Binding of NACP, NACP-112, and NACPδ with the Aβ Peptides Immobilized on a Membrane Filter.** Stock solutions of Aβ at 1.25 mM (Aβ1–28, Aβ1–35, and Aβ25–35 in distilled water and Aβ1–38 in 2-propanol) were prepared immediately prior to each experiment. Two microliters of solution containing 500 pmol of each peptide was spotted onto 1 cm^2 of nitrocellulose membrane and air dried. The filters were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature and washed with PBS for 5 min four times. The filters were successively incubated with 2 μM recombinant NACP, NACP-112, and NACPδ in PBS at 37°C for 16 h, washed with TPBS (PBS containing 0.1% Tween 20) for 5 min four times, incubated with affinity-purified anti-NACP antibody (21) in 1% BSA/TPBS at 4°C for 16 h, washed as described above, and then incubated with ^{125}I-labeled protein A in 1% BSA/TPBS (0.3 μCi/ml; 1 Ci = 37 GBq) at 4°C for 2 h. The NACP bound to the membranes was estimated from the radioactivities quantified by a PhosphorImager (Molecular Dynamics). The background was subtracted, and the average and standard error were calculated (n = 3). To further identify the binding region in Aβ peptides, 2 μM NACP-112 was preincubated with 0, 10, or 50 μM Aβ25–35 or 50 μM Aβ1–28 at 37°C for 16 h. The mixtures were then incubated at 37°C for 16 h with filters on which 500 pmol of Aβ1–38 was spotted. To identify the binding region in NACP-112, V, X1, or Y peptide (5 μM each) was mixed with 2 μM NACP-112. The competing activity of each peptide was assessed as described above.

NACP Binding with Aβ Peptides in Aqueous Solution. Complexes between NACP and Aβ peptides were also detected by immunoblotting. NACP was mixed with various concentrations of Aβ1–38 or Aβ1–35 and incubated at 37°C for 16 or 72 h. After addition of 1/4th vol of 5× sample buffer [0.31 M Tris·HCl, pH 6.8/10% SDS/25 mM EDTA/20% (vol/vol) glycerol/0.1% bromophenol blue], the protein solution was boiled for 5 min and analyzed by immunoblotting.

Thioflavine S Staining of the Aggregated Complex Between NACP and Aβ1–38. The complex between NACP and Aβ1–38 was formed as described above. The precipitate was collected by centrifugation (16,000 × *g* for 10 min) and mixed with thioflavin S (10 mg/ml in H_2O), which had been prepared freshly and filtered through a membrane filter (0.22 μm). The specimen was observed with a fluorescence microscope (Olympus model BHF).

RESULTS

Expression and Purification of NACP. Proteins used for the current study along with peptides V, X1, and Y are schematically presented in Fig. 1*A*. SDS/PAGE of the purified proteins gave single bands (Fig. 1*B*). The sequence of the first 18 amino acid residues was confirmed by gas-phase sequencing (data not shown). Affinity-purified anti-NACP antibody, which was raised against the last 10 amino acid residues of NACP (21), reacted with the purified protein, indicating that the C termini of the proteins were also intact. The purity of the peptides was >95% assessed by HPLC analysis with a Dynamax C_{18} column.

Binding of NACP, NACP-112, and NACPδ to Immobilized Aβ Peptides. Because double staining of senile plaques with anti-NAC antibody and anti-Aβ antibody indicated that NAC and Aβ are both localized to the "core" of plaques as amyloid (20), it is possible that either NAC or NACP binds with Aβ peptide. To test this possibility, we examined the binding activity of NACP to Aβ peptides. NAC binding to Aβ could not be studied because of NAC's strong tendency to self-aggregate (23). As shown in Fig. 2*A*, NACP bound to Aβ1–38 and to a lesser extent to Aβ25–35. On the other hand, binding to Aβ1–28 under the same conditions was not detected, suggesting that the hydrophobic domain of Aβ may be important for NACP binding.

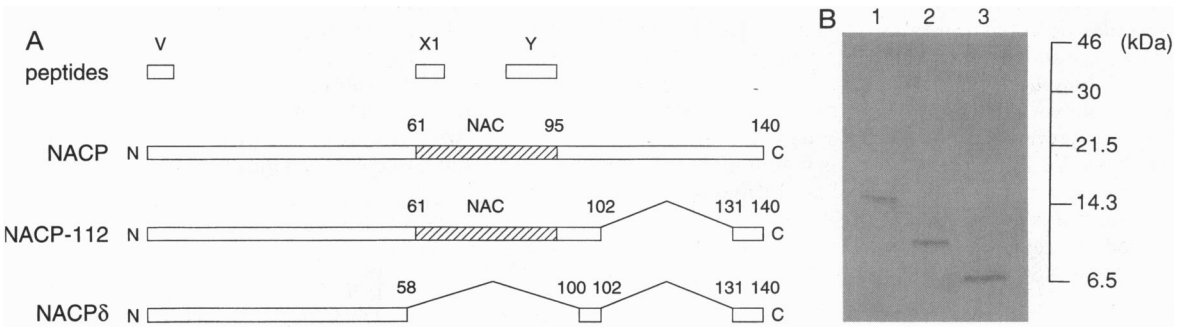

FIG. 1. (*A*) Schematic presentation of NACP, NACP-112, and NACPδ. Positions of peptides are indicated above full-length NACP. (*B*) SDS/PAGE of purified NACP (lane 1), NACP-112 (lane 2), and NACPδ (lane 3).

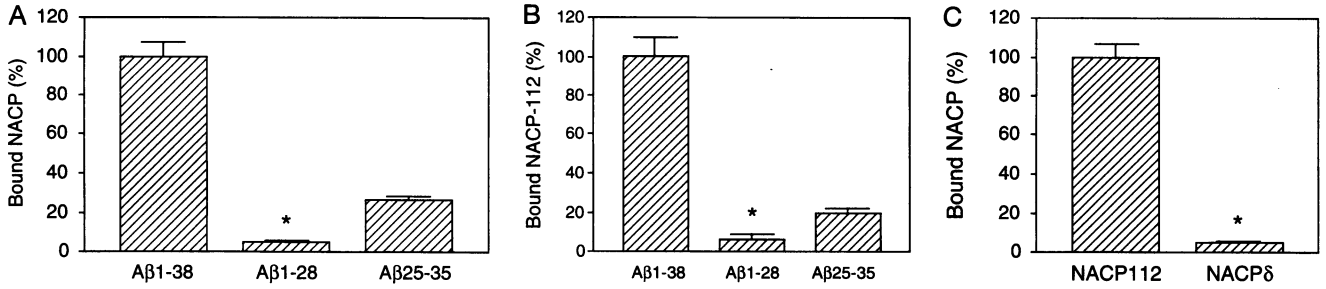

FIG. 2. Binding of NACP, NACP-112, and NACPδ with various Aβ peptides immobilized on membrane filters. Five hundred picomoles of Aβ1–38, Aβ1–28, or Aβ25–35 was spotted onto nitrocellulose membrane filters and incubated with 2 μM NACP (*A*) or NACP-112 (*B*) at 37°C for 16 h. ANOVA with post hoc Newman–Keuls test demonstrates that NACP or NACP-112 binding to Aβ1–28 is significantly lower than that to Aβ1–38 or Aβ25–35 ($P < 0.01$). In a separate set of experiments (*C*), NACPδ binding to Aβ1–38 was compared to NACP-112 binding to Aβ1–38. NACPδ binding was significantly lower than NACP-112 binding by two-tailed Student's *t* test ($P < 0.01$).

Comparable binding was observed between NACP-112 and Aβ peptides. Furthermore, the binding profile of NACP-112 was similar to that of NACP—i.e., NACP-112 bound to Aβ1–38 best among these three peptides. The binding of NACP-112 to Aβ25–35 was 38.2% ± 4.4% that to Aβ1–38. No significant binding was seen between NACP-112 and Aβ1–28 under the same conditions (Fig. 2*B*), confirming the importance of the hydrophobic domain of Aβ in NACP binding.

In a separate set of experiments, bindings of purified NACPδ and NACP-112 to Aβ1–38 were examined by the filter binding assay. As shown in Fig. 2*C*, binding of NACPδ to the Aβ peptide was <5% that of NACP-112, suggesting that the NAC region is largely responsible for binding between NACP and Aβ peptides, possibly through a hydrophobic interaction.

Competition of NACP–Aβ Complex Formation by Peptides. To confirm that the binding region resides within Aβ25–35, a competition assay on filters was carried out. The binding between NACP-112 and Aβ1–38 was inhibited by addition of Aβ25–35 in a dose-dependent manner (Fig. 3*A*). The binding decreased to ≈33% of control in the presence of 50 μM Aβ25–35. On the other hand, Aβ1–28 did not inhibit binding at the same concentration, suggesting that NACP binds to Aβ1–38 mainly through the Aβ25–35 region, whereas the surrounding sequence is necessary for maximum binding.

To identify a region within NACP-112 responsible for binding with Aβ peptides, the filter binding assay was carried out in the presence or absence of synthetic NACP peptides as competitors. First, it was determined that inclusion of the N-terminal peptide V did not affect NACP-112 binding to Aβ (data not shown). Then, NACP-112 was mixed with peptide V, X1, or Y and incubated with filters onto which Aβ1–38 had been immobilized. The C-terminal portion of NAC represented by peptide Y interfered with the binding of NACP-112 with Aβ1–38, whereas the N-terminal portion of NAC, peptide X1, did not affect binding (Fig. 3*B*).

Analysis of NACP–Aβ Complex. NACP binding with Aβ peptides in a solid-phase filter assay prompted us to investigate its binding activity to the Aβ peptides in the aqueous phase. Unincubated NACP migrated between 14 and 16 kDa on PAGE (Fig. 4, lanes 1). A larger molecular mass band at ≈35 kDa appeared after incubation at 37°C for 16 h (Fig. 4*A*, lane 2) or for 72 h (Fig. 4 *B* and *C*, lane 2). As there was no protein other than NACP present in the incubation mixture, it is likely that this ≈35-kDa band was the SDS-resistant complex of the NACP dimer. The inclusion of Aβ in the NACP solution seemed to increase (Fig. 4*A*, lanes 2 and 3) and then reduce the intensity of the NACP dimer band in a 16-h incubation in a dose-dependent manner (Fig. 4*A*, lanes 3–5). This apparent reduction of the NACP dimer band in the presence of high concentrations of Aβ may be due to the shift of the band to a more diffused and slightly larger molecular mass band. The shift in the mobility of the NACP dimer band is more clearly seen in a 72-h incubation (Fig. 4*B*, lanes 3–5). A 72-h incubation of NACP with 125-fold excess Aβ produced a band of 20 kDa, presumably the 1:1 complex of NACP–Aβ that was SDS resistant (Fig. 4*B*, lane 5). The appearance of the band at 45 kDa at the same time, however, suggested the presence of the complex between the NACP dimer and Aβ dimer as well (Fig. 4*B*, lane 5). In a separate set of experiments (Fig. 4*C*), 20 pmol of NACP was mixed with 2.5 nmol of Aβ1–38, incubated at 37°C for 72 h, and analyzed by Western blotting with anti-NACP antibody. No NACP bands were detected on the blot

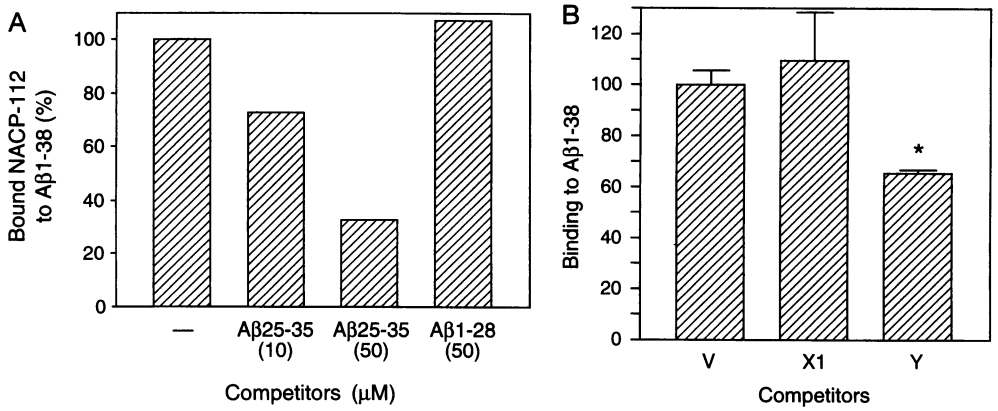

FIG. 3. Identification of the binding region in NACP-112 and Aβ1–38 by competition assay. (*A*) Specific binding of NACP-112 to Aβ1–38 was quantified and expressed with the binding of NACP-112 to Aβ1–38 in the absence of a competitor set to 100. Values are averages of two independent experiments that did not differ by more than 20%. (*B*) Filter binding assay was carried out in the presence of competing NACP peptides. *, $P <$ 0.01 (ANOVA with post hoc Newman–Keuls test). Separate experiments demonstrated that NACP binding to Aβ1–38 in the presence of V peptide was identical to that without competing peptide.

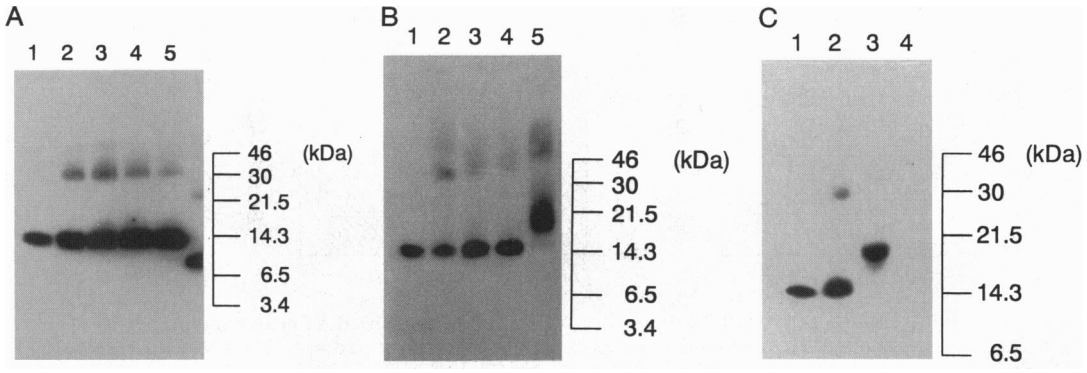

FIG. 4. Dose-dependent formation of a complex between NACP and Aβ. Twenty picomoles of NACP was incubated with 0–2.5 nmol of Aβ1–35 at 37°C for either 16 h (*A*) or 72 h (*B*). The proteins were analyzed by immunoblotting. Lanes 1, 20 pmol of NACP with no incubation. The same amount of NACP was incubated with either 0 pmol (lanes 2), 100 pmol (lanes 3), 500 pmol (lanes 4), or 2.5 nmol (lanes 5) of Aβ1–35. In a separate set of experiments (*C*), the complex between NACP and Aβ1–35 and Aβ1–38 peptides was analyzed. Twenty picomoles of NACP was mixed with 2.5 nmol each of either Aβ1–38 or Aβ1–35 at pH 7.3. Mixtures were incubated at 37°C for 72 h. Lane 1, NACP with no incubation; lanes 2–4, NACP incubated without Aβ (lane 2), with Aβ1–35 (lane 3), or with Aβ1–38 (lane 4). Note that the experiments in *B* (lane 5) and *C* (lane 3) are under identical conditions, but because of the lighter exposure the ≈45-kDa band is weaker in *C* (lane 3).

probably because a large SDS-insoluble complex was formed, which could not enter into the gel (Fig. 4*C*, lane 4). The control experiments with Aβ1–35 produced an SDS-insoluble complex between Aβ1–35 and NACP (Fig. 4*C*, lane 3).

Formation of Aβ Aggregates Triggered by NACP in Aqueous Solution. To test the possibility of large aggregate formation from Aβ1–38, the following experiment was performed. A stock solution of Aβ1–38 at 1.25 mM was diluted with PBS, and 2.5 nmol of the peptide was mixed with 20 pmol of NACP at pH 7.3 in a total vol of 20 μl. As shown in Fig. 5*A*, <30% of Aβ1–38 was recovered in the supernatant when incubated in the presence of NACP, whereas 100% of Aβ1–38 was recovered in the supernatant when incubated without NACP, suggesting that NACP stimulated aggregation of Aβ1–38.

Amyloid can be detected by thioflavin S staining (2). Fig. 5*B* demonstrates that the thioflavin S-positive aggregates were formed in the presence of NACP. No aggregates were detected when incubation was carried out with Aβ alone or with NACP alone under the same conditions.

DISCUSSION

NACP bound to Aβ1–38 and Aβ25–35 immobilized on nitrocellulose, but it did not bind to Aβ1–28 under the same conditions. NACP-112 also bound to Aβ1–38 and Aβ25–35, whereas it did not bind to Aβ1–28 under the same conditions (Fig. 2). Furthermore, the binding between NACP-112 and Aβ1–38 was inhibited by Aβ25–35 but not by Aβ1–28 (Fig. 3). These data strongly suggest that the domain of Aβ responsible for binding to NACP is the Aβ25–35 region. These observations are interesting since molecules such as apolipoprotein E, α_1-antichymotrypsin, and complement C1q were reported to bind to the Aβ1–28 region (Fig. 6).

The carboxyl half of the NAC region may play an important role in its binding with Aβ. NACPδ with a deletion in the NAC domain did not bind Aβ. Furthermore, the binding between NACP-112 and Aβ1–38 immobilized on membrane filters was inhibited by addition of peptide Y, but not by peptide X1, demonstrating that at least the last 15 residues of the NAC region are involved in the binding (Figs. 3*B* and 6). Thus, our data demonstrate that NACP can bind to Aβ peptides to form SDS-insoluble complexes through interaction between the last 15 residues of NAC and the Aβ25–35 region, which was reported to be toxic to neuronal cells (24, 25). The direct demonstration of NAC binding to Aβ was reported recently (26).

Many types of cells have been found to release Aβ peptide in a soluble form under physiological conditions (4–6). How-

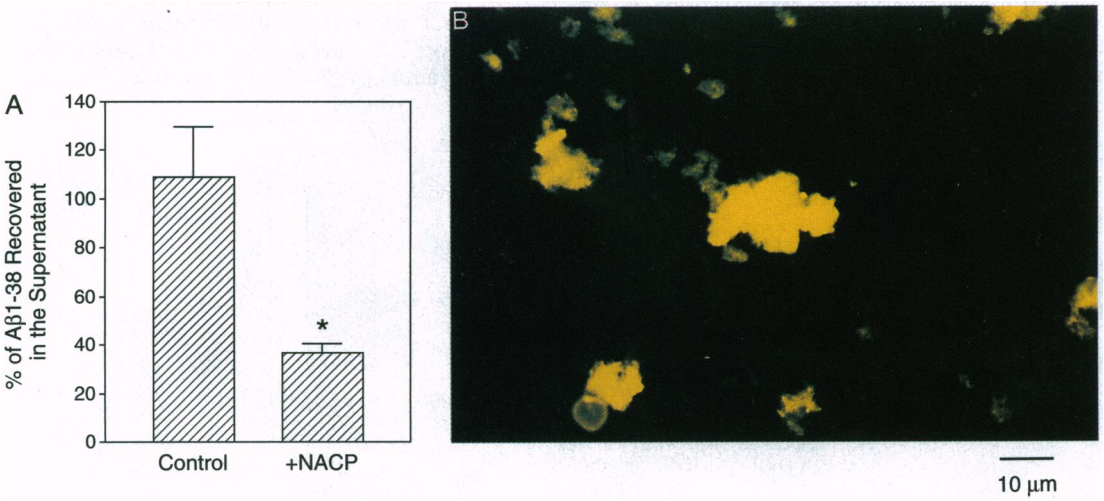

FIG. 5. Stimulation of Aβ1–38 aggregation by NACP. Two and one-half nanomoles of Aβ1–38 was mixed in PBS with 20 pmol of NACP at pH 7.3 and incubated at 37°C for 72 h. As control, Aβ1–38 was incubated in PBS at pH 7.3. After incubation, the mixture was centrifuged at 16,000 × *g* for 30 min. Two microliters of each supernatant as well as nontreated peptide were spotted onto nitrocellulose filter, and the amounts of the peptide were measured by immunodot blotting. (*A*) Amount of Aβ peptide in the supernatant was estimated from the radioactivities recovered on dots. *, $P < 0.01$ by two-tailed Student's *t* test. (*B*) Aβ1–38 aggregates formed in the presence of a trace amount (0.8%) of NACP were stained with thioflavine S.

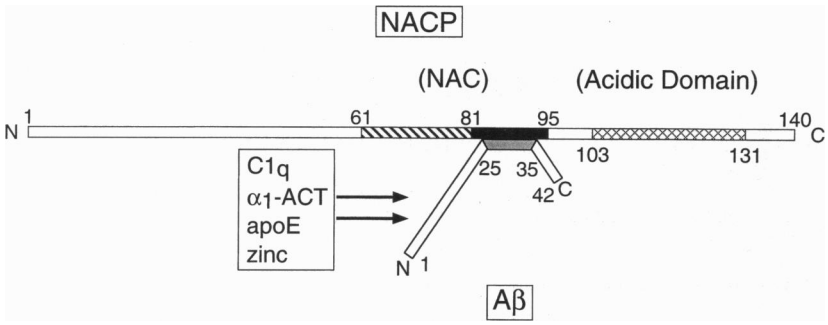

FIG. 6. Binding of NACP with Aβ. The binding domain of Aβ for complement C1q, α_1-antichymotrypsin (α1-ACT), apolipoprotein E (apoE), and zinc is Aβ1–28. On the other hand, NACP binds to Aβ25–35. Hatched and solid boxes in NACP represent the NAC region (amino acids 61–95); cross-hatched box represents the acidic domain (amino acids 103–130) missing in NACP-112.

ever, the presence of Aβ is not correlated with AD. Therefore, it is likely that there are conditions, in addition to the presence of Aβ, that need to be met for amyloid to be formed. The production of a longer form of Aβ (amino acids 1–42 instead of 1–40) may be critical in a familial case of AD (27). However, this does not seem to apply to sporadic cases of AD (28). Posttranslational modifications of Aβ affect the rate of Aβ aggregation, but the presence of these modifications needs to be tested in the brain tissue of AD patients (29, 30). More recently, it was found that a high concentration of zinc, α_1-antichymotrypsin, and apolipoprotein E can cause Aβ conversion to amyloid (18, 31). The current data suggest that binding between the secreted Aβ peptide and the trace amount of NACP might also be a step that stimulates amyloid formation. Our immunocytochemical work, as well as the homology search, indicated that NACP is a presynaptic protein colocalized with synaptophysin (21). It is intriguing to note that APP, the precursor protein of Aβ, is also localized to the growing axon terminals and the presynaptic terminals (32). Thus, metabolic alterations of neurons may cause abnormal release of presynaptic proteins, which in turn form amyloid. Although APP is rather ubiquitously distributed throughout the brain and body, NACP is localized preferentially to the brain, and its distribution closely coincides with the affected brain regions rich in plaque amyloid in AD (21). Thus, the available data are consistent with the idea that NACP may be involved in initiation of amyloid pathology in AD, whereas APP provides the material for the amyloid pathology to develop.

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